

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth

MARIA NESTEROVA & YOON S. CHO-CHUNG

Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute,
National Institutes of Health, Building 10, Room 5B-05, Bethesda, Maryland 20892-1750, USA

Correspondence should be addressed to Y.S.C.-C.

Expression of the RI_α subunit of cAMP-dependent protein kinase type I is enhanced in human cancer cell lines, in primary tumours, in cells after transformation and in cells upon stimulation of growth. We have investigated the effect of sequence-specific inhibition of RI_α gene expression on *in vivo* tumour growth. We report that single injection RI_α antisense treatment results in a reduction in RI_α expression and inhibition of tumour growth. Tumour cells behaved like untransformed cells by making less protein kinase type I. The RI_α antisense, which produces a biochemical imprint for growth control, requires infrequent dosing to halt neoplastic growth *in vivo*.

Standard cytotoxic chemotherapy for cancer is usually accompanied by systemic toxicity. The ratio of the toxic dose to the therapeutic dose is relatively low, reflecting the large number of cellular targets affected by the chemotherapeutic agent. In principle, because of the specificity of Watson-Crick base pairing, an antisense oligonucleotide targeted at a gene involved in the neoplastic cell growth should interfere only with that gene's expression, resulting in arrest of cancer cell growth.

Enhanced expression of the RI_α subunit of cAMP-dependent protein kinase (PKA)¹ has been shown in human cancer cell lines and in primary tumours, as compared with normal counterparts, in cells after transformation with the Ki-ras oncogene or transforming growth factor- α , and upon stimulation of cell growth with granulocyte-macrophage colony-stimulating factor (GM-CSF) or phorbol esters^{2,3}. Conversely, a decrease in the expression of RI_α correlates with growth inhibition induced by site-selective cAMP analogues in a broad spectrum of human cancer cell lines⁴.

There are two types of PKA, type I (PKA-I) and type II (PKA-II), which share a common C subunit but contain distinct R subunits, RI and RII, respectively⁵. Through biochemical studies and gene cloning, four isoforms of the R subunits, RI_α, RI_β, RII_α and RII_β, have been identified^{6,7}. Three distinct C subunits, C_α (ref. 7), C_β (refs 8, 9) and C_γ (ref. 10) have also been identified; however, preferential coexpression of one of these C subunits with any of the R subunits has not been found^{9,10}. The R isoforms differ in tissue distribution^{11,12} and in biochemical properties¹³. The two general isoforms of the R subunit also differ in their subcellular localization. RI is found throughout the cytoplasm, whereas RII localizes to nuclei, nucleoli, Golgi apparatus and the microtubule-organizing center^{2,14,15}. The expression of RI/PKA-I and RII/PKA-II has an inverse relationship during ontogenic development and cell differentiation^{2,3}. However, the significance of the presence of these two isoforms of PKA in the biological functions of cAMP has not been determined.

We hypothesize that the RI_α is an ontogenic growth-inducing protein and that its constitutive expression disrupts normal

ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy. The two general classes of PKA R subunits, RI and RII, have conserved amino acid sequences at the carboxyl terminus but differ significantly at the amino terminus¹⁶. An RI_α antisense phosphorothioate oligodeoxynucleotide corresponding to the N-terminal 8–13 codons of RI_α was constructed (RI_α antisense). We then investigated the effect of sequence-specific inhibition of RI_α gene expression on *in vivo* tumour growth.

Results

Inhibition of tumour growth

A single subcutaneous (s.c.) injection into nude mice bearing LS-174T human colon carcinoma with RI_α antisense resulted in almost complete suppression of tumour growth for 7 days as assessed either by tumour volume (Fig. 1a) or by tumour weight (Fig. 1b). There was no apparent sign of toxicity as evaluated by hematocrit levels, body weight and food intake. Even after 14 days, tumour growth was significantly inhibited in the antisense-treated animals (Fig. 1b). In contrast, tumours in saline-treated animals showed continued growth (Fig. 1), and tumours in untreated or control antisense-treated animals grew at a rate similar to those in saline-treated animals. The results of tumour growth inhibition were confirmed with three additional RI_α antisense constructs (each of the 21-base polymers directed to codons 1–7, 14–20 and 94–100, respectively, of human RI_α), which previously have been shown to inhibit growth in a variety of human cancer cell lines^{17,18}.

We also did experiments using the RI_α 8–13 codon antisense two-base-pair-mismatched oligonucleotide (5'-GCG-CGC-CTC-CTC-GCT-GGC-3') to substantiate further the specificity of the antisense oligonucleotide. The data revealed that antisense mismatched oligonucleotide was unable to inhibit tumour growth. (The average tumour weights (in milligrams) \pm s.d. at 12 days post-treatment for mismatched oligonucleotide, scrambled oligonucleotide, and antisense oligonucleotide were 1533 ± 250 ($n = 5$), 1490 ± 305 ($n = 5$), and 439 ± 88 ($n = 5$), respectively.)

The LS-174T human colon carcinoma in this model behaved in a very aggressive manner. Within a week after tumour cell inoculation, a palpable tumour mass was formed, and thereafter tumours often killed host animals in 4–5 weeks. The results of these experiments provide clear evidence that the $R_{I\alpha}$ antisense phosphorothioate oligodeoxynucleotide has significant antitumour activity in an *in vivo* model system, where tumour growth was arrested for up to 14 days of observation after a single dose of antisense.

The human $R_{I\alpha}$ antisense (directed to 8–13 codons of human $R_{I\alpha}$) also inhibited proliferation of the *c-ras*^H-transformed mouse fibroblast cell line (K. Noguchi and Y.S.C.-C., unpublished results). The control antisense had no effect on the growth of the transformed fibroblasts. The human and mouse $R_{I\alpha}$ 8–13 codons contain one mismatched nucleotide at the 5' end followed by a stretch of 10 nucleotides that are 100% homologous and contain four mismatches at the 3' end^{12,19}. Although the human $R_{I\alpha}$ 8–13 codons are not 100% homologous with the corresponding codons of the mouse $R_{I\alpha}$, the antisense directed to this human sequence was apparently able to hybridize with the mouse $R_{I\alpha}$ mRNA, inducing growth inhibition. Thus, the lack of systemic toxicity in the $R_{I\alpha}$ antisense-treated animals was not due to the inability of the $R_{I\alpha}$ antisense to cross-react with the mouse $R_{I\alpha}$ gene.

Downregulation of $R_{I\alpha}$

We examined whether the $R_{I\alpha}$ antisense could specifically decrease the amount of $R_{I\alpha}$ subunit in tumours (Fig. 2). At 0, 16 and 24 hours, 2, 3, 5 and 7 days after the single injection of $R_{I\alpha}$ antisense, the animals were killed, and tumours were analysed for the amount they contained of each of the R subunits of PKA. Tumour extracts were photoaffinity labelled with 8-N₃-[³²P]cAMP and then immunoprecipitated with the monospecific antibodies anti- $R_{I\alpha}$, - $R_{II\alpha}$ and - $R_{II\beta}$ (kindly provided by S.D. Park, Seoul National University, Seoul, South Korea), and the immunoprecipitated proteins were resolved

by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)²⁰. The $R_{I\alpha}$ levels in tumours from the antisense-treated animals were markedly decreased within 24 hours and remained at low levels (10–20% of that in tumours from saline-treated animals) for up to 2–3 days (Fig. 2). Specific targeting of $R_{I\alpha}$ by the antisense is evident since $R_{II\alpha}$ levels remained unchanged (Fig. 2). At 5–7 days post-antisense treatment, the $R_{I\alpha}$ levels in tumours were elevated to levels similar to those in tumours from saline-injected animals (Fig. 2). At day 3 after antisense treatment, tumours that contained unreduced amounts of $R_{I\alpha}$ contained a new species of R, $R_{II\beta}$, along with a reduced amount of $R_{II\alpha}$ (Fig. 2). The increase in $R_{II\beta}$ expression was also found in tumours that contained decreased levels of $R_{I\alpha}$ without reduction in $R_{II\alpha}$ content (data not shown). $R_{II\beta}$ appeared 24 hours to 3 days post-antisense treatment but was not detected in control tumours (saline or control antisense-treated). These data show that the antisense-targeted suppression of $R_{I\alpha}$ brought about a compensatory increase in $R_{II\beta}$ levels. Similar observations were made previously in cultured cancer cell lines upon treatment with $R_{I\alpha}$ antisense^{17,18}.

Immunoprecipitated $R_{I\alpha}$ and $R_{II\beta}$ show a doublet, a major fast mobility band and a minor slow mobility one (Fig. 2). The doublets were specific bands because the addition of 1,000-fold excess cold cAMP added in the experiments using photoaffinity labelling with immunoprecipitation resulted in the disappearance of both bands. The slow mobility band of the $R_{I\alpha}$ doublet may be $R_{I\alpha}$, which is highly homologous with $R_{I\alpha}$ but has a higher molecular weight than $R_{I\alpha}$ (ref. 12) and that the $R_{II\beta}$ doublet may represent the autophosphorylated form of $R_{II\beta}$ (ref. 21).

Elimination of PKA-I with induction of PKA-II_β

Upon antisense treatment, the level of $R_{I\alpha}$ after its initial suppression for a few days, subsequently increased in tumours (Fig. 2). In cells, $R_{I\alpha}$ can exist either in its subunit form or in the form of the PKA-I holoenzyme. As the $R_{I\alpha}$ subunit can act as a

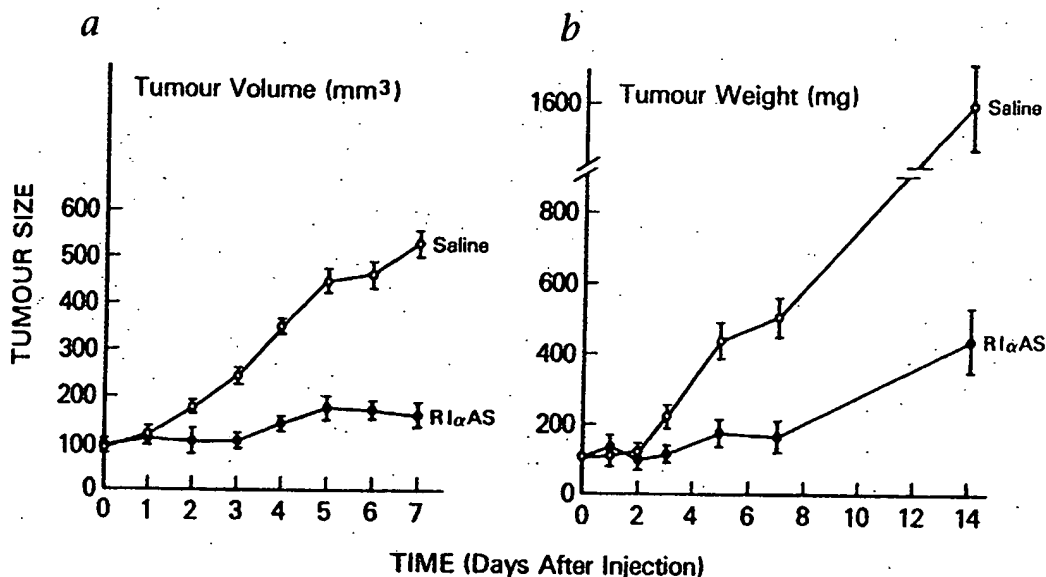


Fig. 1 Inhibition of *in vivo* tumour growth by a single dose of $R_{I\alpha}$ antisense. *a*, Tumour volume obtained from daily measurement. *b*, Tumour weight at the time mice were killed. Data represent means \pm s.d. of 8–32 tumours in *a*; mean \pm s.d. of 8 tumours in *b*. These data were obtained from four separate experiments where each experiment used 24 mice.

cAMP sink, $R_{I\alpha}$ in the holoenzyme complex may be of functional importance. We therefore examined whether $R_{I\alpha}$ antisense could influence the PKA isozyme distribution in tumours. We subjected tumour extracts to diethylaminoethyl (DEAE) ion-exchange chromatography, and fractions were assayed for PKA activity and cAMP-binding activity. Saline-treated control tumours showed two major peaks of PKA activity that were coincident with peaks of cAMP-binding activity. PKA-I eluted between 50 and 100 mM NaCl, whereas PKA-II eluted between 220 and 300 mM NaCl (Fig. 3, 24 hours post saline injection). In addition, there were two minor cAMP-binding peaks with no PKA activity eluted at 130 and 330 mM NaCl, respectively (Fig. 3). These were identified as $R_{I\alpha}$ and $R_{II\alpha}$ subunits, respectively, by photoaffinity labelling with 8-N₃-[³²P]cAMP (Fig. 4). Control tumours (24 hours to 7 days post saline injection or control antisense treatment) contained PKA-I and PKA-II in a 1:2 ratio (Fig. 3). The antisense treatment completely eliminated PKA-I, the $R_{I\alpha}$ -containing holoenzyme, and the $R_{I\alpha}$ subunit, from tumours within 24 hours (Fig. 3). This downregulation of PKA-I lasted for up to 5–7 days post-antisense treatment (Fig. 3) even when the $R_{I\alpha}$ levels increased until they reached the levels of control tumours (Figs. 2 and 3). This indicates that the $R_{I\alpha}$ that increased subsequently to its initial suppression after antisense treatment was mostly present in its subunit form rather than in its holoenzyme form, PKA-I.

Earlier reports demonstrated that brain and heart PKA-II eluted at slightly different salt concentrations from DEAE cellu-

lose columns⁴. The brain contains high levels of $R_{II\alpha}$, whereas the heart expresses the $R_{II\alpha}$ isoform. Heterogeneity of PKA-II has been reported and was attributed to either $R_{II\alpha}$ trimer formation²⁴ or a $R_{II\alpha}$ tetramer with nonsaturating amounts of bound cAMP²⁵. Otten *et al.*²⁶ identified three peaks of PKA-II in *ras*-transformed NIH3T3 (R3T3) cells overexpressing either mouse $R_{II\alpha}$ or rat $R_{II\alpha}$; the first and third peaks of PKA-II were associated with $R_{II\alpha}$ and $R_{II\beta}$, respectively, and the second peak contained a mixture of $R_{II\alpha}$ and $R_{II\beta}$. Retroviral vector-mediated overexpression of the human $R_{II\alpha}$ or $R_{II\beta}$ gene in LS-174T colon carcinoma cells has also shown three peaks of PKA-II; the first PKA-II is associated with $R_{II\alpha}$, and the second and third peaks contain mainly $R_{II\beta}$.²⁷

Concomitant with the suppression of PKA-I, the antisense brought about changes in the PKA-II profile of tumours. The control tumours contained PKA-II with its main peak eluted at 260 mM NaCl (Fig. 3). Within 24 hours after antisense treatment, PKA-II with its peak eluted at 220 mM NaCl appeared prominently (Fig. 3). This altered PKA-II profile persisted in tumours for up to 2–3 days after antisense treatment, but by day 5, the PKA-II profile became similar to that of control tumours (Fig. 3). Photoaffinity labelling of the R subunits from tumours 24 hours after antisense treatment with 8-N₃-[³²P]cAMP showed the presence of $R_{II\alpha}$ only in fractions 33–37 (Figs 3 and 4). Immunoprecipitation of the R subunits confirmed their identity by photoaffinity labelling. Thus, PKA-II eluted at 220 mM NaCl (Fig. 3) was PKA-II_α. $R_{II\alpha}$ and PKA-II_β were detected in 50% of the tumours analysed 24 hours to 3 days after

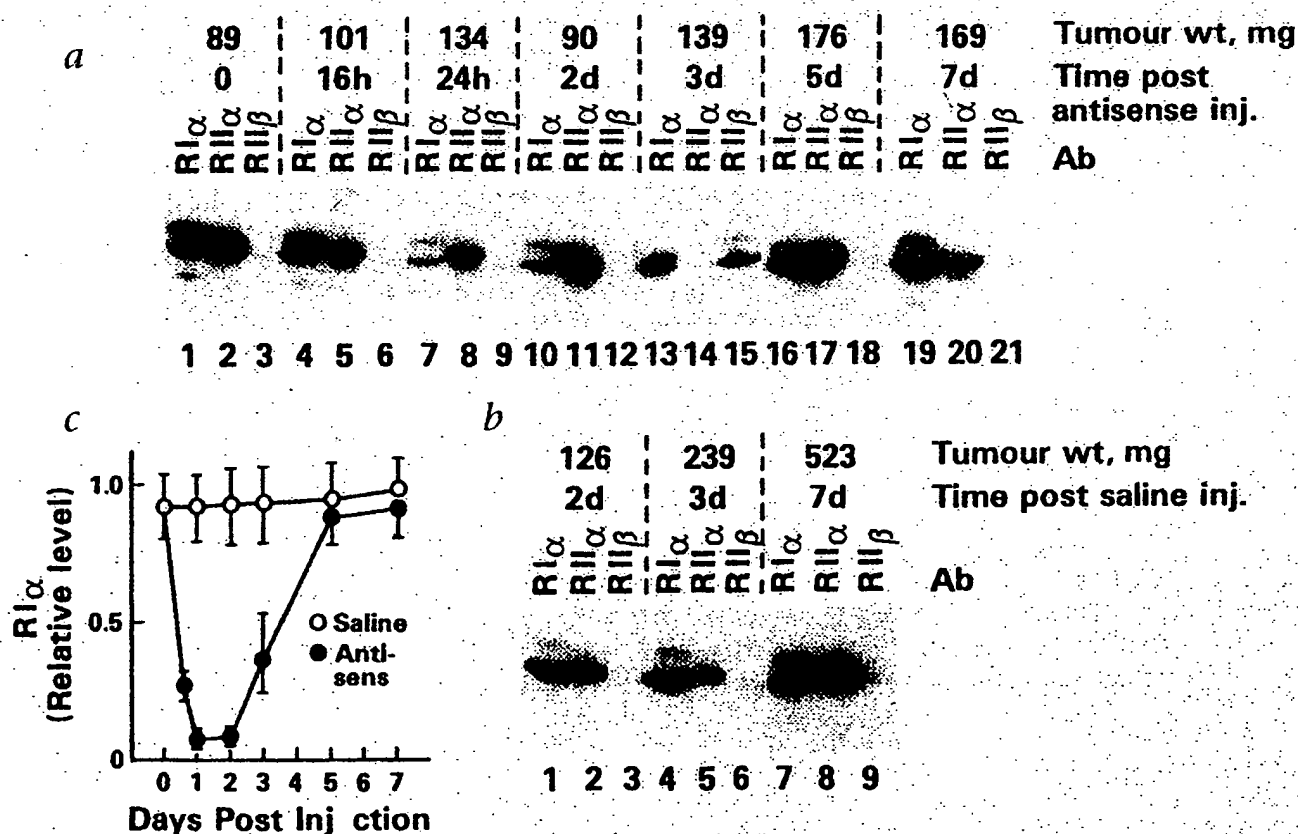


Fig. 2 Suppression of $R_{I\alpha}$ levels in tumours by a single dose of $R_{I\alpha}$ antisense. The R subunit levels in tumours were determined by photoaffinity labelling followed by immunoprecipitation. Gels in *a* and *b* represent one of four separate experiments that gave similar results. *c*, Quantification of $R_{I\alpha}$ levels by densitometric tracings of autoradiographs (*a* and *b*): the data represent relative average values \pm s.d. of four experiments.

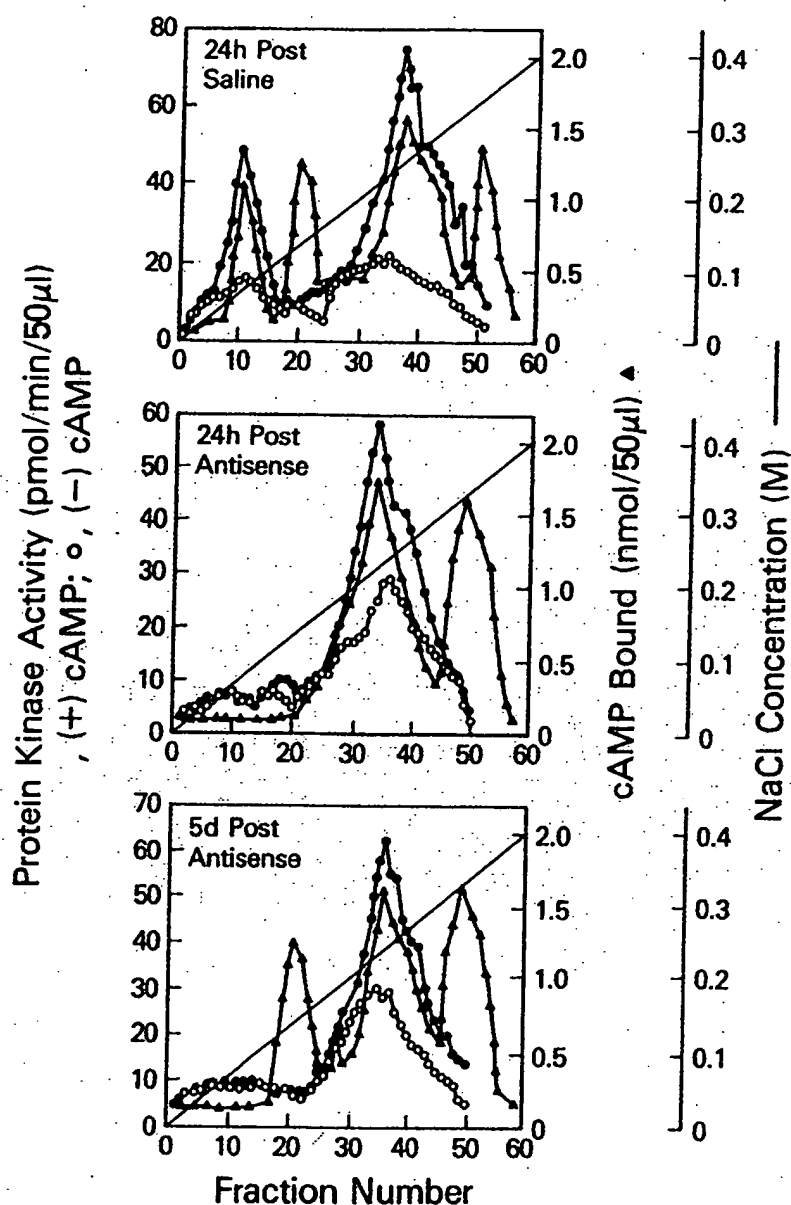


Fig. 3 PKA-I downregulation by a single dose of RI_{α} antisense. PKA activity in the absence (○) or presence (●) of $5 \mu M$ cAMP and cAMP-binding activity (▲) was measured in DEAE column eluents. Each chromatograph was repeated 3 or 4 times and yielded similar elution profiles.

antisense treatment but were not detected in control tumours (saline injection or control antisense treatment). These data suggest that the C subunits of PKA in tumours are in equilibrium between PKA-I and PKA-II and that deprivation of RI_{α} and PKA-I by the antisense led to an increase in PKA-II through the induction of PKA-II.

Increase of R subunit proteolysis

The RII_{α} expression in these tumours was accompanied by an increase in a low-molecular-weight R species, probably the proteolytically degraded R subunits^{4,28} (Fig. 4). This apparent increase in R proteolysis was not an experimental artifact of tumour homogenization, because the homogenization buffer contained a cocktail of protease inhibitors. Furthermore, the low-molecular-weight R species were frequently detected in tu-

mours 24 hours to 3 days after antisense treatment but were never observed in control tumours (saline or control antisense treated). Thus, the antisense treatment triggered RI_{α} downregulation as well as R subunit proteolysis, though the mechanism of these actions is not yet clear.

Discussion

We have demonstrated that a single injection of RI_{α} antisense resulted in an acute reduction in RI_{α} expression and a sustained inhibition of tumour growth. This antisense-inhibition of tumour growth was confirmed by the use of four distinct antisense constructs corresponding to the different N-terminal regions of RI_{α} codons. The growth inhibition may have been due to actions other than the blockage of RI_{α} expression, as nonspecific binding of the oligonucleotide or its degradation products to biological targets has been shown²⁹. As discussed below, however, our data show that the antisense-inhibition of RI_{α} expression and modulation of protein kinase A isozymes are clearly related to the inhibition of tumour growth. Thus, at most, nonspecific binding of oligonucleotide probably played a minimal role in the observed growth inhibition.

In vivo pharmacokinetics studies in rodents showed a single intravenous dose of phosphorothioate oligodeoxynucleotide leaves the vascular space after 2–3 hours (phase α $t_{1/2} = 15$ –25 min), and its elimination from the body, which is almost completely urinary, requires 72 hours (phase β $t_{1/2} = 20$ –40 hours)³⁰. Our results are in accordance with such pharmacokinetics of oligonucleotide and show a single s.c. dose of RI_{α} antisense produces an acute reduction in RI_{α} ($t_{1/2} RI_{\alpha} = 31$ hours (ref. 31)) content within 24 hours, and thereafter for 2–3 days. This reduction in RI_{α} triggered a compensatory increase in RII_{α} , and an elimination of PKA-I activity.

The downregulation of PKA-I lasted for several days even after the RI_{α} suppression ceased, suggesting that RI_{α} may be functionally different, because it no longer formed the holoenzyme, PKA-I. We speculate this may be due to the following: (1) once RI_{α} is downregulated, the free C subunits complex with all of the available RII_{α}

subunits to form PKA-II_α; (2) the remaining free C subunits trigger the synthesis of RII_{α} and form PKA-II_α; (3) RII_{α} has a greater half-life than RI (RII_{α} , $t_{1/2} = 125$ hours; RI , $t_{1/2} = 31$ hours (ref. 31)); therefore, once RII_{α} is synthesized, it remains in the cell for a longer time and favours complex formation with the C subunit as compared with RI ; (4) the subsequently formed RI_{α} , after its initial suppression (due to the antisense), can no longer form PKA-I holoenzyme in the presence of the increased amount of PKA-II, which is favoured over PKA-I in its holoenzyme formation³; and (5) RI may be degraded and cannot form PKA-I. Although the exact mechanisms of action await future studies, our results showed that the antisense produced a biochemical imprint in tumour cells. The cells behaved like untransformed cells by making less PKA-I than PKA-II. This may be the basis for the suppression of tumour growth.

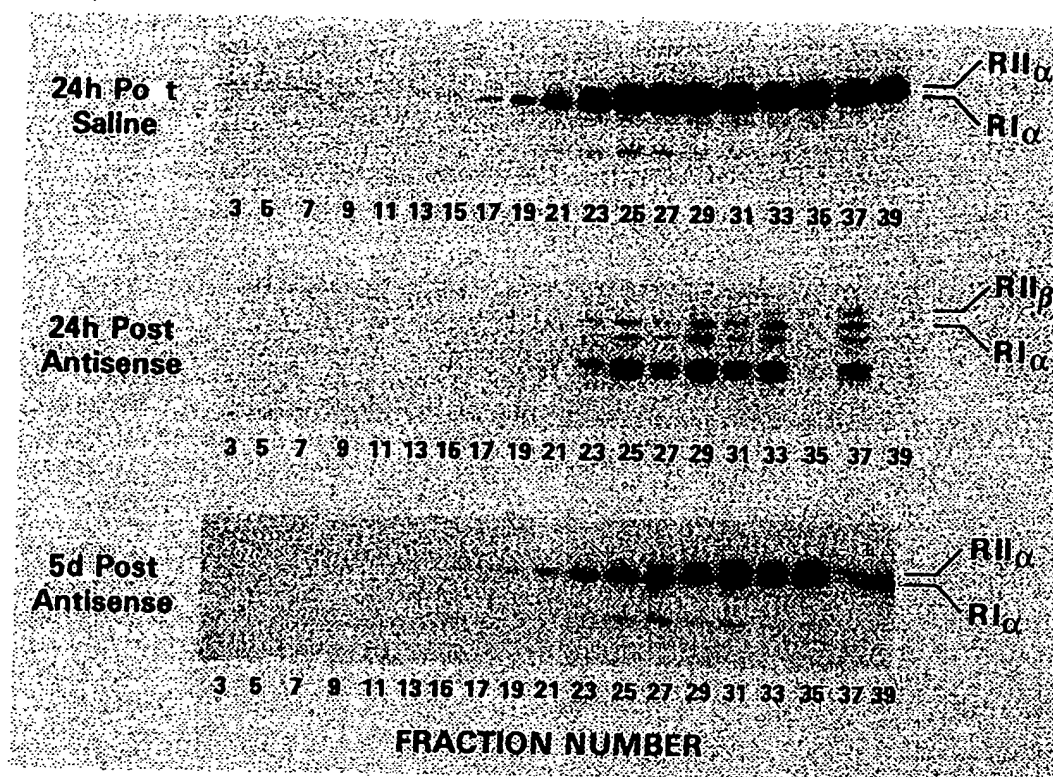


Fig. 4 PKA R subunit distribution in DEAE column fractions of tumours from the antisense- and saline-treated animals. The R subunit isoforms in DEAE column eluents (Fig. 3) were measured by photoaffinity labelling with 8-N₃-[³²P]cAMP and SDS-PAGE²². The data represent one of four separate experiments that gave similar results.

We have demonstrated that a single dose of RI_α antisense triggered the suppression of RI_α that preceded tumour growth inhibition. Importantly, the growth inhibition persisted, even after RI_α suppression ceased, as long as PKA-I (the RI_α-containing holoenzyme) downregulation was present. The single-injection antisense treatment introduced a programming in growth control in tumour cells, and thus was sufficient to produce a sustained inhibition of growth. This unexpected finding has a great impact on the application of antisense oligonucleotides as therapeutic agents, especially in terms of potency, targeting and cost. Our results suggest that an antisense like RI_α antisense, which is capable of producing a biochemical imprint for growth control in tumour cells, may require relatively infrequent repetitive dosing to maintain its inhibitory effect toward tumour growth *in vivo*.

Methods

Tumour growth and antisense treatment. LS-174T human colon carcinoma cells (1×10^6 cells) were inoculated s.c. into the left flank of athymic mice. The RI_α antisense phosphorothioate oligodeoxynucleotide (corresponding to the RI_α NH₂-terminus 8–13 codons (RI_α antisense) (5'-GCC-TGC-CTC-CTC-ACT-GCC-3')) and control antisense (the same base composition as the RI_α antisense with the sequence jumbled) were kindly provided by T. Geiser (Lynx Therapeutics, Hayward, California). A single dose of RI_α antisense or control antisense (1 mg per 0.1 ml saline per mouse) or saline (0.1 ml per mouse) was injected s.c. into the right flank of mice when tumour size reached 80–100 mg, 1 week after cell inoculation. Tumour volumes were obtained from daily measurement of the longest and shortest diameters and calculation by the formula, $4/3\pi r^3$ where $r = (\text{length} + \text{width})/4$. At each indicated time, two animals from the control and antisense-treated groups were killed, and tumours were removed, weighed, immediately frozen in liquid N₂, and kept frozen at -80°C until used.

Photoaffinity labelling followed by immunoprecipitation of R subunits. The tumours were homogenized with a Teflon/glass homogenizer in ice-cold buffer 10 (Tris-HCl, pH 7.4, 20 mM; NaCl, 100 mM; NP-40, 1%; sodium deoxycholate, 0.5%; MgCl₂, 5 mM; pepstatin, 0.1 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.2 mM; aprotinin, 0.4 mg ml⁻¹; and soybean trypsin inhibitor, 0.5 mg ml⁻¹; filtered through a 0.45- μm pore size membrane), and centrifuged for 5 min in an Eppendorf microfuge at 4°C . The supernatants were used as tumour extracts.

The amount of R subunits of PKA in tumours was determined by photoaffinity labelling with 8-N₃-[³²P]cAMP followed by immunoprecipitation with the R antibodies as previously described²⁰.

DEAE-cellulose column chromatography. Extracts (10 mg protein) of tumours from antisense-, control antisense- or saline-treated animals were loaded onto DEAE cellulose columns (1 \times 10 cm) and fractionated with a linear salt gradient²². PKA activity was determined in the absence or presence of 5 μM cAMP²². cAMP-binding activity was measured by the method described previously and expressed as the specific binding²³.

Acknowledgements

We thank R.I. Glazer, A. Budillon, S. Abrams and J.W. Greiner for critical reading of the manuscript; T. Geiser for providing the phosphorothioate oligodeoxynucleotides; S.D. Park for providing antiserum to RI_α, RII_α and RII_β proteins; B. Chaney for technical assistance; and G.R. Lee for the sequence homology analysis of human and mouse RI_α gene.

RECEIVED 13 DECEMBER 1994; ACCEPTED 20 APRIL 1995

1. Krebs, E.G. Protein kinase. *Curr. Topics Cell Regul.* 5, 99–133 (1972).
2. Lohmann, S.M. & Walter, U. Regulation of the cellular and subcellular concentrations and distribution of cyclic nucleotide-dependent protein kinases. In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, vol. 18, 63–117 (Raven, New York, 1984).
3. Cho-Chung, Y.S. Role of cyclic AMP receptor proteins in growth, differentiation, and suppression of malignancy: New approaches to therapy [Perspec-

- tives in cancer research]. *Cancer Res.* 50, 7093-7100 (1990).
4. Beebe, S.J. & Corbin, J.D. Cyclic nucleotide-dependent protein kinases. In *The Enzymes: Control by Phosphorylation*, vol. 17, part A, 43-111 (Academic, New York, 1986).
 5. McKnight, G.S. *et al.* Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. *Recent Prog. Horm. Res.* 44, 307-335 (1988).
 6. Levy, F.O. *et al.* Molecular cloning, complementary deoxyribonucleic acid structure and predicted full-length amino acid sequence of the hormone-inducible regulatory subunit of 3',5'-cyclic adenosine monophosphate-dependent protein kinase from human testis. *Molec. Endocrinol.* 2, 1364-1373 (1988).
 7. Uhler, M.D. *et al.* Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. *Proc. natn. Acad. Sci. U.S.A.* 83, 1300-1304 (1986).
 8. Uhler, M.D., Chivlia, J.C. & McKnight, G.S. Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. *J. biol. Chem.* 261, 15360-15363 (1986).
 9. Showers, M.O. & Maurer, R.A. A cloned bovine cDNA encodes an alternate form of the catalytic subunit of cAMP-dependent protein kinase. *J. biol. Chem.* 261, 16288-16291 (1986).
 10. Beebe, S.J. *et al.* Molecular cloning of a unique tissue-specific protein kinase (Cy) from human testis—representing a third isoform for the catalytic subunit of the cAMP-dependent protein kinase. *Molec. Endocrinol.* 4, 465-475 (1990).
 11. Øyen, O. *et al.* A unique mRNA species for a regulatory subunit of cAMP-dependent protein kinase is specifically induced in haploid germ cells. *FEBS Lett.* 229, 391-394 (1988).
 12. Clegg, C.H., Cadd, G.G. & McKnight, G.S. Genetic characterization of a brain-specific form of the type I regulatory subunit of cAMP-dependent protein kinase. *Proc. natn. Acad. Sci. U.S.A.* 85, 3703-3707 (1988).
 13. Cadd, G.G., Uhler, M.D. & McKnight, G.S. Holoenzymes of cAMP-dependent protein kinase containing the neural form of type I regulatory subunit have an increased sensitivity to cyclic nucleotides. *J. biol. Chem.* 265, 19502-19506 (1990).
 14. Kapoor, C.L. & Cho-Chung, Y.S. Compartmentalization of regulatory subunits of cyclic adenosine 3',5'-monophosphate-dependent protein kinases in MCF-7 human breast cancer cells. *Cancer Res.* 43, 295-302 (1983).
 15. Nigg, E.A., Schäfer, G., Hiltz, H. & Eppenberger, H.M. Cyclic-AMP-dependent protein kinase type II is associated with the golgi complex and with centrosomes. *Cell* 41, 1039-1051 (1985).
 16. Taylor, S.S. *et al.* cAMP-dependent protein kinase: Prototype for a family of enzymes. *FASEB J.* 2, 2677-2685 (1988).
 17. Yokozaki, H. *et al.* An antisense oligodeoxynucleotide that depletes RI₁ subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. *Cancer Res.* 53, 868-872 (1993).
 18. Tortora, G., Yokozaki, H., Pepe, S., Clair, T. & Cho-Chung, Y.S. Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase. *Proc. natn. Acad. Sci. U.S.A.* 88, 2011-2015 (1991).
 19. Sandberg, M., Tasken, K., Øyen, O., Hansson, V. & Jahnsen, T. Molecular cloning, cDNA structure and deduced amino acid sequence for A type I regulatory subunit of cAMP-dependent protein kinase from human testis. *Biochem. biophys. Res. Commun.* 149, 939-945 (1987).
 20. Tortora, G., Clair, T. & Cho-Chung, Y.S. An antisense oligodeoxynucleotide targeted against the type RI₁ regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects. *Proc. natn. Acad. Sci. U.S.A.* 87, 705-708 (1990).
 21. Rosen, O.M. & Erlichman, J. Reversible autophosphorylation of a cyclic 3',5'-AMP-dependent protein kinase from bovine cardiac muscle. *J. biol. Chem.* 250, 7788-7794 (1975).
 22. Rohlf, C., Clair, T. & Cho-Chung, Y.S. 8-Cl-cAMP induces truncation and down-regulation of the RI₁ subunit and up-regulation of the RI₂ subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. *J. biol. Chem.* 268, 5774-5782 (1993).
 23. Tagliaferri, P., Katsaros, D., Clair, T., Neckers, L., Robins, R.K. & Cho-Chung, Y.S. Reverse transformation of Harvey murine sarcoma virus-transformed NIH/3T3 cells by site-selective cyclic AMP analogs. *J. biol. Chem.* 263, 409-416 (1988).
 24. Connelly, P.A., Hastings, T.G. & Reimann, E.M. Identification of a ternary complex between cAMP and a trimeric form of cAMP-dependent protein kinase. *J. biol. Chem.* 261, 2325-2330 (1986).
 25. Cobb, C.E., Beth, A.H. & Corbin, J.D. Purification and characterization of an inactive form of cAMP-dependent protein kinase containing bound cAMP. *J. biol. Chem.* 262, 16566-16574 (1987).
 26. Otten, A.D., Parenteau, L.A., Døskeland, S. & McKnight G.S. Hormonal activation of gene transcription in *ras*-transformed NIH3T3 cells overexpressing RI₁ and RI₂ subunits of the cAMP-dependent protein kinase. *J. biol. Chem.* 266, 23074-23082 (1991).
 27. Nesterova, M., Budillon, A., Pepe, S., Cereseto, A. & Cho-Chung, Y.S. Introducing an autophosphorylation site mutation in the RI₁ regulatory subunit of cAMP-dependent protein kinase abolishes the RI₁-mediated regulatory function. *FASEB J.* 8, A1328 (1994) (abstract).
 28. Srivastava, A.K. & Stellwagen, R.H. Presence of the sites for interacting with cyclic AMP and with catalytic subunit on small fragments of protein kinase regulatory subunit. *J. biol. Chem.* 253, 1752-1755 (1978).
 29. Wagner, R.W. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 372, 333-335 (1994).
 30. Iversen, P. *In vivo* studies with phosphorothioate oligonucleotides: Pharmacokinetics prologue. *Anti-Cancer Drug Des.* 6, 531-538 (1991).
 31. Weber, W. & Hiltz, H. cAMP-dependent protein kinases I and II: Divergent turnover of subunits. *Biochemistry* 25, 5661-5667 (1986).